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Original Paper

Isolation and proteomic analysis of rice Golgi membranes: cis-Golgi membranes labeled with GFP-SYP31

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Abstract The Golgi membranes labeled with cis-Golgi marker GFP-SYP31 were isolated from suspension-cultured cells of rice transformed with *35S::GFP-SYP31* by floating through a discontinuous sucrose density gradient in the presence of 5 mM $MgCl_2$. The specific fluorescence intensity of final membrane preparation increased to approximately 150-fold in comparison with that of the post-nucleus soluble fraction. Specific activity of membrane-bound α-mannosidase (cis-Golgi) markedly increased, but NDPase (medial-/trans-Golgi) and NADPH-cytochrome c reductase endoplasmic reticulum were weakly detected in the membrane fraction. The other organelle marker enzymes, cytochrome c oxidase (mitochondria), alkaline pyrophosphatase (plastid), and catalase (peroxisome) were not detectable. Comparative display of protein spots in GFP-SYP31-labeled membranes, microsomal membranes and soluble fraction on the two dimensional gels showed that some proteins are markedably concentrated in the cis-Golgi membrane fraction. Furthermore, the mass spectrometric analysis of proteins separated by SDS-polyacrylamide gel electrophoresis indicated that the highly purified Golgi membranes contained several membrane traffic-related proteins and ER resident proteins. These findings indicated a close relationship between the ER and the cis-Golgi membranes.

Key words: Cis-Golgi, green fluorescent protein, small GTPase, rice, syntaxins 31.

The plant Golgi complex is a multifunctional organelle, responsible for the biosynthesis of complex cell-surface polysaccharides, the processing and modification of glycoproteins, and the sorting station of polysaccharides and proteins destined for different locations. In contrast to the clustered Golgi stacks of mammalian cells that form a juxta- or perinuclear network (Rambourg and Clermont 1997), plant Golgi stacks are dispersed singly or in small clusters throughout the cytoplasm and show stop-and-go tumbling movements along actin microfilaments, which run in parallel to the endoplasmic reticulum (ER) network (Boevink et al. 1998; Nebenführ et al. 1999; Hawes and Satiat-Jeunemaitre 2005). Morphologically, a Golgi stack is composed of a series of flattened cisternae that exhibit a distinct polarity. This polarity reflects the underlying vectorial organization of the stack, which receives products, particularly proteins, from the ER at the cis face and exports them after processing and maturing from the trans face (Staehelin et al. 1995). Complex polysaccharides, which are synthesized exclusively in the Golgi cisternae, are also assembled in a cis-to-trans direction (Moore et al. 1991).

Recently, there is controversy over the existence of an ER-Golgi complex which appears to be the structural and functional link between the ER and Golgi bodies (Nebenführ et al. 1999). The majority of plant COPI and COPII machinery including small GTPases and fusion proteins (SNAREs) has been cloned (Contreras et al. 2000; Pimpl et al. 2000; Phillipson et al. 2001). They are well conserved throughout the eukaryotic kingdom and their identification supported that the vesicle carrier theory operates in plant (Sanderfoot et al. 1999; Nebenführ et al. 2002). On the other hand, membrane traffic between the ER and the Golgi bodies has been shown to be reversible and cytoskeleton-independent (Brandizzi et al. 2002). Furthermore, live-cell imaging and developments in electron microscopic studies have revealed the possibility of a continuum between the ER

Abbreviations: ARF, ADP ribosylation factor; BFA, brefeldin A; ER, endoplasmic reticulum; ESI, electrospray ionization; GFP, green fluorescent protein; IEF, isoelectric focusing; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; SNARE, soluble N-ethyl-maleimide sensitive factor attachment protein receptors; SYP31, syntaxins 31; TFA, trifluoroacetic acid; YPT, yeast protein transport.
This article can be found at http://www.jspcmb.jp

and Golgi (Brandizzi et al. 2002; Yang et al. 2005). Moreover, ER membranes and cis-Golgi membranes reacted similarly to many cytochemical agents, suggesting a close biochemical relationship (Képès et al. 2005). To clarify the structural and functional relationship between the ER and Golgi, we need to establish the isolation procedure of the cis-Golgi compartment.

Previously, we succeeded in the separation of distinct compartments of Golgi complex from suspension-cultured cells of rice employing a modified discontinuous sucrose density gradient centrifugation technique (Mikami et al. 2001). Furthermore, proteomic analysis of the Golgi-IDPase fraction was carried out, however, surprisingly, members of intracellular traffic were not identified in the proteins separated and detected in 2D-gels (Tanaka et al. 2004). In the present study, we attempted to isolate and analyze the cis-Golgi membranes from suspension-cultured cells of rice transformed with the cis-Golgi marker GFP-SYP31 (Uemura et al. 2004; Pratelli et al. 2004).

Materials and methods

Plant materials

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were supplied from the Niigata Agricultural Research Institute (Niigata, Japan).

Binary vector constructions and plant transformations

The *XbaI-KpnI* fragment released from pGFP-SYP31 (Uemura et al. 2004) was inserted into the multi-cloning sites of the binary vectors pZH2B-Tnos (Hajdukiewicz et al. 1994) to create pZH2B-35S-GFP-SYP31. The vector was transformed into the competent cells of *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) treated with 20 mM $CaCl_2$. *Agrobacterium*-mediated transformation and regeneration of rice plant were performed according to the methods described by Asatsuma et al. (2005). The plants were eventually transferred to soil in pots and grown to maturity in a greenhouse. Rice cells were cultured as described previously (Mitsui et al. 1996). Growth and morphological characteristics of the transgenic cells were indistinguishable from those of the wild-type plant.

Introduction of plasmids into onion cells

pGFP-SYP31 and pST-mRFP (sialyl transferase fused to the monomeric red fluorescent protein; Kim et al. 2001, Latijnhouwers et al. 2005, Matsuura-Tokita et al. 2006) were introduced into onion epidermal cells by the particle bombardment method using a helium-driven particle accelerator (PDS-1000/He; BIO-RAD) with all basic adjustments set as recommended by the manufacturer. Three μg plasmid DNA in 10 μl distilled water was mixed with 10 μl of 60 mg ml^{-1} gold particles (1.0 μm in diameter) solution, 10 μl of 2.5 mM $CaCl_2$ and 4 μl of 0.1 M spermidine, and incubated for 30 min at room temperature. Gold particles coated with plasmid DNA were rinsed with cold ethanol, and then suspended with 10 μl ethanol gently. The gold particles were bombarded twice into onion cells using the particle delivery system with 1100 psi rupture discs.

Microscopic analysis

The transgenic rice cells expressing GFP-SYP31 were sectioned with Vibratome (VIB3000 plus-SKR, Meiwa) into 25-μm thick slices, treated with or without 90 μM BFA in Murashige-Skoog medium for 90 min, and immediately observed by confocal laser scanning microscopy (FV1000, Olympus) according to the manufacturer's protocol. The bombarded onion epidermal cells were subjected to the fluorescence microscope system, composed of a microscope (Olympus BX-61), a cooled CCD camera (Hamamatsu, EM-CCD) and an imaging software Lumina Vision (Mitani). The fluorescence of GFP was imaged by excitation at 470 to 490-nm and detection at 510 to 550-nm, and that of RFP was imaged by excitation at 520 to 550-nm and detection at ≧580 nm. In onion cells, 20 to 30 images per cell, from the top to bottom of the cell with 2–5 μm apart, were taken and combined into one image.

Isolation of Golgi membranes

Cells suspension-cultured for 6 days were gently homogenized with a half volume of 25 mM HEPES-KOH (pH 7.0), 1 mM EDTA and 0.5 M mannitol. The homogenate was passed through two layers of gauze and centrifuged at 1,000×g for 20 min and 10,000×g for 30 min, sequentially. The supernatant was layered on a 15% (w/w) sucrose layer and 50% sucrose cushion containing 25 mM HEPES-KOH (pH 7.0) and further centrifuged at 100,000×g for 3 h. The membrane fraction trapped on the 50% sucrose cushion was used as the microsome preparation.

The microsome preparation was adjusted to 42% sucrose and 5 mM $MgCl_2$, and then applied to bottom of discontinuous sucrose gradient consisting of 26, 30, 34, and 38% sucrose containing 25 mM HEPES-KOH (pH 7.0) and 5 mM $MgCl_2$. The gradients were centrifuged at 100,000×g for 3 h. The GFP-SYP31-labeled cis-Golgi membranes were floated boundary phase between 34% and 38% sucrose layer. The peak fraction of GFP-SYP31-labeled membranes (fraction no. 9 in Figure 3A) was adjusted to 42% sucrose again and subjected to the second discontinuous sucrose gradient centrifugation/floating.

After centrifugation, each fraction collected from the top of the tube was subjected to assays and blotting analyses. The sucrose content was determined with a refractometer (Atago, NAT-1T). All procedures were performed at 4°C.

Assays

Enzyme activities were measured according to the methods described in the accompanying references: UDP-glucose pyrophosphorylase (Kimura et al. 1992), alkaline pyrophosphatase (Rodríguez-López et al. 2000), cytochrome c oxidase, catalase, NDPase and NADPH-cytochrome c reductase (Mitsui et al. 1990), α-mannosidase (Kishimoto et al. 2001), GlcNAc transferase (Mikami et al. 2001). Protein contents were determined as described by Bradford (Bradford

et al. 1976). The fluorescence of GFP was measured at 484 nm excitation and 510 nm emission with a spectrofluorophotometer (Shimadzu RF-5300PC).

1D- and 2D-PAGE

The microsomal and Golgi membranes diluted with equal volume of 25 mM HEPES-NaOH (pH 7.0) were centrifuged at 100,000×*g* for 30 min. The pellet was resuspended with 25 mM HEPES-NaOH (pH 7.0) and 2% (w/v) CHAPS, and sonicated for 10 min in an ice bath. The suspension was mixed with one tenth volume of 100% trichloroacetic acid, and the precipitate was suspended and washed with 100% ethanol three times. The resultant precipitate was suspended with lysis buffer containing 9 M urea, 3% (w/v) IGEPAL CA-630, and 2% (v/v) 2-mercaptethanol at room temperature, and then centrifuged at 100,000×*g* for 30 min to remove insoluble materials. The supernatant was used as the solubilized membrane proteins.

1D-PAGE: An aliquot of the solubilized membrane proteins was applied to SDS-PAGE with 12% separation gel.

2D-PAGE: 2D-PAGE was performed using IEF-PAGE and SDS-PAGE according to the procedure of Mikami et al. (2001) with a slight modification. The cytosolic and solubilized membrane proteins (200 μg) with 2% (v/v) ampholine (pH 3.5–10.0) were applied to the cathode side of first-dimensional disc gel (11.5 cm×φ3 mM) composed of 8 M Urea, 4% (w/v) acrylamide, 1% IGEPAL CA-630, 2% ampholine (pH3.5–10), 0.02% ammonium persulfate, and 0.003% *N,N,N′,N′*-tetramethyl ethylenediamine. The electrophoresis was carried out at 300 V for 20 h, followed by 600 V for 30 min at 4°C. After IEF, the gel was placed in an equilibration solution containing 0.06 M Tris-HCl (pH 6.8), 2.5% (w/v) SDS, 5% (w/v) 2-mercaptoethanol and 10% (w/v) glycerol for 10 min at room temperature, and then subjected to SDS-PAGE. Protein spots on the gels were visualized with Coomassie brilliant blue R250 (CBB). Isoelectric point and molecular size of each protein was estimated using 2D SDS-PAGE standards (Bio-Rad). The localization sites and quantity of individual proteins were evaluated by using image analysis software, Image Master 2D Elite (Amersham Pharmacia Biotech) and PDQuest (Bio-Rad).

Immunoblots

Procedure of immunoblotting was identical to the previous report (Mikami et al. 2001). Anti-GFP antibodies (Invitrogen) and peroxidase-conjugated anti-rabbit IgG IgG (EY Laboratory) were diluted to 1 : 100 and 1 : 500, respectively.

Mass analysis

Protein bands excised from either CBB-stained 1D- or 2D-gel were washed with 25% methanol and 7% acetic acid for 12 h at room temperature, and further destained with 50 mM NH_4HCO_3 in 50% methanol at 40°C for 1 h. After removing the destained solution carefully, the gels were rinsed with H_2O twice and dried in Speed-Vac. The gels were swollen and incubated with 10 mM dithiothreitol, 100 mM NH_4HCO_3 at 60°C for 1 h. The dried gels were swollen again and incubated with 40 mM iodoacetamide, and 100 mM NH_4HCO_3 at room temperature for 30 min in the dark. The gels were rinsed twice with H_2O, crashed, and then dried again in Speed-Vac. The gel pieces were re-swollen and incubated with 50 μl of 20 nM trypsin (Promega) and 10 mM Tris-HCl (pH 8.0) at 37°C for 12 h. Peptide fragments were extracted and collected from the gels with 0.03% (w/v) TFA/33% (v/v) acetonitrile, 0.1% TFA, 0.1% TFA/50% acetonitrile, 0.1% TFA in acetonitrile, sequentially. The obtained peptides were subjected to MALDI-TOF-MS (Shimadzu, AXIMA-CFR,). The matrix solution was prepared by mixing a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 100% acetonitrile (1 : 1, v/v). The peptides were further analyzed by electrospray ionization (ESI) Q-TOF MS/MS (Micromass, Q-Tof Micro) according to the manufacturer's protocol. The obtained peptide mass fingerprints were analyzed by Mascot search system (Matrix Science, http://www.matrixscience.com).

Results and discussion

Production of rice cells stably expressing cis-Golgi marker GFP-SYP31

SYP31/AtSED5 belongs to a family of SNARE (soluble N-ethyl-maleimide sensitive factor attachment protein receptors), Qa-SNAREs/Syntaxins (Sanderfoot et al. 2000; Bock et al. 2001). The transient expression of GFP-SYP31 in *Arabidopsis* suspension-cultured cells with or without Brefeldin A (BFA) has demonstrated that GFP-SYP31 localizes at the face of cis-Golgi (Uemura et al. 2004). To isolate and characterize rice cis-Golgi membranes, we produced transgenic rice plants expressing a cis-Golgi marker GFP-SYP31. In the transgenic rice cells, the fluorescence from GFP-SYP31 was distributed in intracellular dotted structures (Figure 1A, control). Transgenic cells treated with BFA showed a distribution of the GFP fluorescence changed to the ER network structures (Figure 1A, BFA). Thus, the Golgi bodies were visualized with GFP-SYP31 in rice cells. Furthermore, the cis-Golgi marker GFP-SYP31 and the trans-Golgi marker ST-mRFP (Kim et al. 2001; Latijnhouwers et al. 2005) were transiently and simultaneously expressed in a monocotyledonous onion epidermal cells. The fluorescence of GFP-SYP31 was usually separated clearly from, but occasionally existed in very close proximity to, that of ST-mRFP (Figure 1B). These results confirm that the cis-Golgi membranes were labeled with GFP-SYP31 in monocotyledonous cells. We used the suspension-cultured cells of transgenic rice for isolating Golgi membranes. The growth and morphological characteristics of transgenic rice cells were indistinguishable from the wild-type plant (not shown), indicating that the overexpression of GFP-SYP31 exhibits no toxicity for the structure and function of rice cells.

Isolation of GFP-SYP31-labeled Golgi membranes from suspension-cultured cells

The isolation procedure of GFP-SYP31-labeled Golgi membranes is summarized in Figure 2. Microsomal

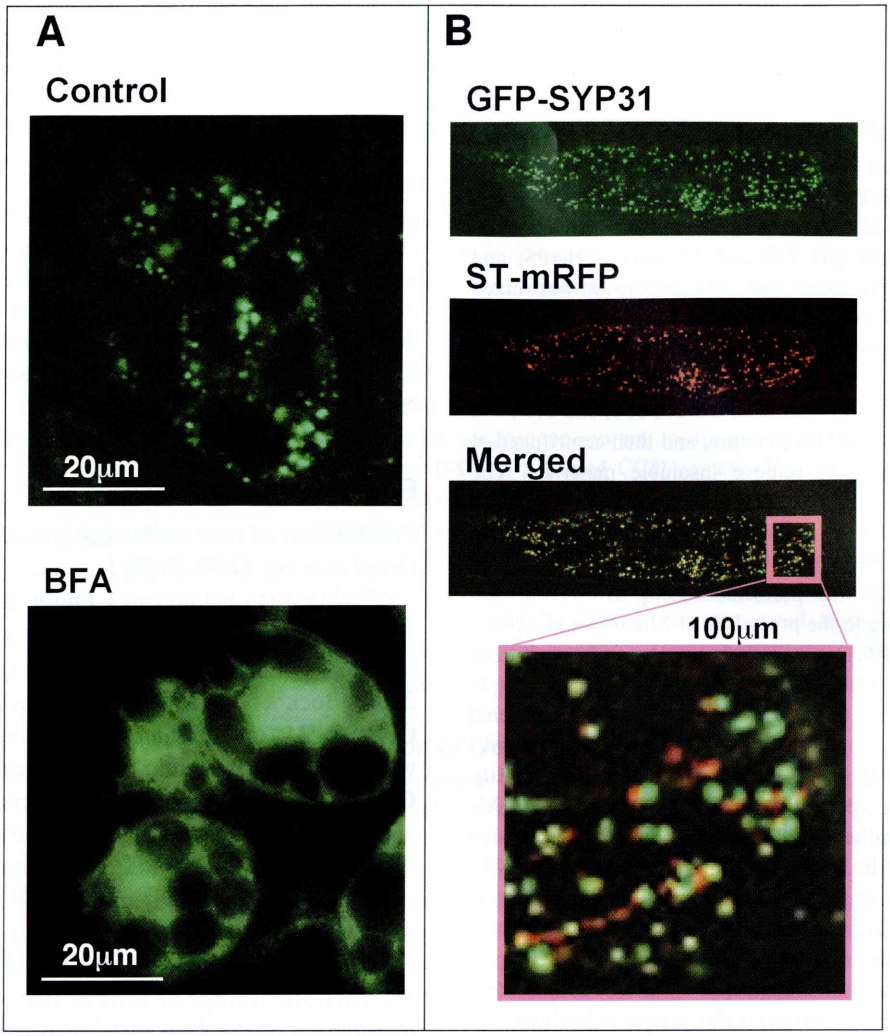

Figure 1. Fluorescence images in rice and onion epidermal cells expressing cis-Golgi marker GFP-SYP31. (A) Rice cells stably expressing GFP-SYP31 were incubated with or without 90 μM BFA for 90 min at 25°C. Upper panel, control; lower panel, BFA treatment. (B) Onion epidermal cells were simultaneously bombarded with pGFP-SYP31 and pST-mRFP. Fluorescence of both GFP-SYP31 and ST-mRFP showed a similar dotted structures, which occasionally existed in very close proximity to each other.

membranes prepared from suspension-cultured rice cells expressing GFP-SYP31 subjected to discontinuous sucrose density gradient centrifugation/floating, the GFP-SYP31-labeled membranes were constantly distributed at the interface between 34 and 38% sucrose in both the first and second centrifugation/floating (Figure 3). The specific fluorescence intensity of the final membrane preparation from the second centrifugation/floating was increased to approximately 27-fold in comparison with that of microsomal membranes (Table 1). In order to further evaluate its purity, the other organelle and cytosol markers were traced during the isolation steps. The ER marker enzymes NADPH-cytochrome c reductase, plastidial alkaline pyrophosphatase, mitochondrial cytochrome c oxidase, peroxisomal catalase and cytosolic UGPase were not or very weakly detectable in the final preparation of Golgi membrane (Table 1). A Golgi marker α-mannosidase tightly bound to the membrane was concentrated in the Golgi (GFP-SYP31) fraction, whereas the level of the other Golgi maker enzyme NDPase was very low. Mannosidase I has been reported to localize in the cis-Golgi (Nebenführ et al. 1999) and NDPase is mainly distributed in the medial- and trans-Golgi (Robinson and Kristen 1982). Thus, the GFP-SYP31-labeled Golgi membrane fraction was shown to be cis-Golgi enriched, highly purified from the other organelles and cytosol.

Characterization of GFP-SYP31-labeled Golgi membrane proteins

Soluble, microsomal and GFP-SYP31-labeled Golgi membrane proteins prepared from the transgenic rice cells were subjected to 2D-PAGE. The result of differential display on 2D-gels showed that some specific proteins were concentrated in the Golgi membrane fraction (Figure 4). Major protein spots (no. 1–10) on the

2D-gels were analyzed by peptide mass finger printing, these indicating to be glyceraldehyde-3-phosphate dehydrogenases (GAPDH), voltage-dependent anion channels (VDAC), and ABA-inducible proteins r40g2 and r40c1 (Table 2). They were not typical Golgi proteins, however, a proteomic study of animal Golgi membranes revealed that GAPDH and VDAC might be associated with the Golgi membranes (Morel et al. 2000). Furthermore, biochemical and cell biological studies have provided evidence that GAPDH interacts with a G-protein Rab2 and plays an essential role in ER to Golgi transport in mammalian NRK cell (Tisdale et al. 2004), and a VDAC isoform localizes in the secretory pathway through the Golgi to the cell membrane in other mammalian COS 7 cell (Buettner et al. 2000). Whether or not GAPDH and VDAC are involved in the functions of plant Golgi remains to be determined.

Estimation of protein contents from the visualized 2D-

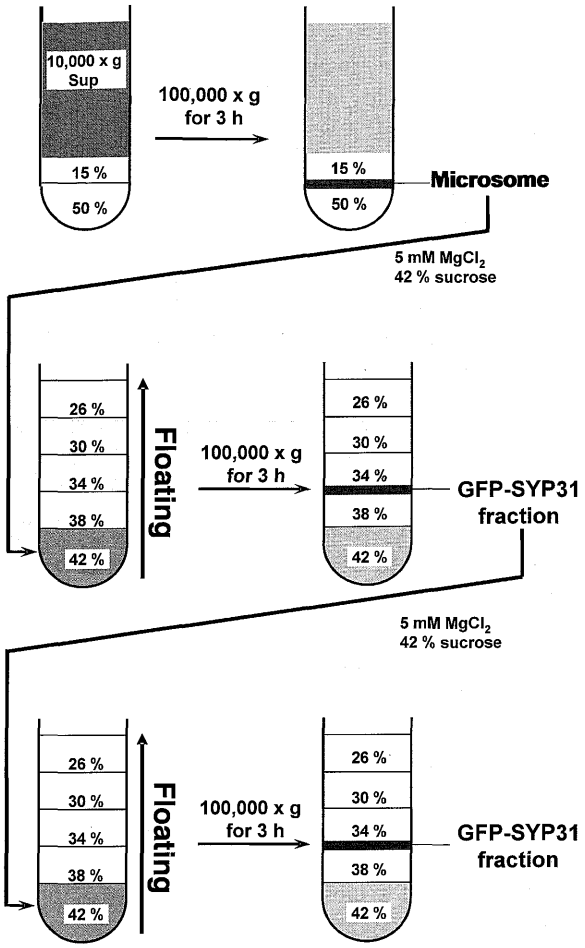

Figure 2. Schematic illustration of fractionation of GFP-SYP31-labeled Golgi membranes from rice cells. Microsome membrane preparation was applied to the bottom of discontinuous sucrose gradient consisting of 26, 30, 34, and 38% sucrose. The GFP-SYP31-labeled Golgi membranes floated in the boundary phase between 34% and 38% sucrose layer by centrifugation. The membrane fraction was subjected again to the second discontinuous sucrose gradient centrifugation/floating. Details of fractionation procedure are described in the text.

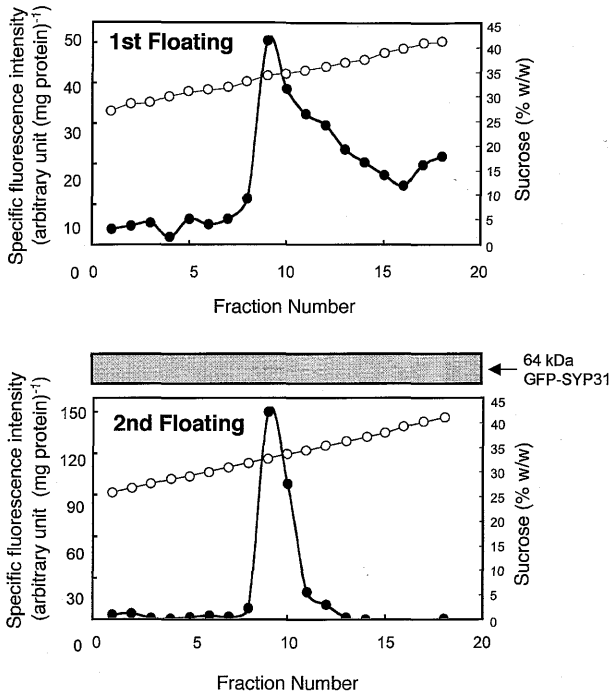

Figure 3. Separation profiles of GFP-SYP31-labeled Golgi membranes after the first and second discontinuous sucrose gradient centrifugation/floating. An aliquot of each fraction separated by the second centrifugation/floating was subjected to immunoblotting with anti-GFP antibodies and peroxidase-conjugated second antibodies. The results of immunoblots were shown in top of 2nd floating panel. (-●-), specific fluorescence intensity of GFP-SYP31 (see Table 1). (-○-), sucrose concentration.

Table 1. Purity of GFP-SYP31-labeled Golgi membranes

Marker enzymes	PNS*	Microsomes	Golgi (GFP-SYP31)
GFP-SYP31	1**	5.62	150.0
(Specific fluorescence intensity: arbitrary unit/ mg protein)			
α-Mannosidase (nmol/min/mg)	-	6.8	36.6
NDPase (μmol/min/mg)	-	0.957	0.097
NADPH-cyt.c reductase (nmol/min/mg)	1.28	2.98	0.008
Alkaline pyrophosphatase (nmol/min/mg)	21.4	0.14	b.d.l.***
Cyt.c oxidase (μmol/min/mg)	0.55	0.047	b.d.l.
Catalase (μmol/min/mg)	0.102	0.071	b.d.l.
UGPase (nmol/min/mg)	95	50	7.1

*PNS, post-nucleus soluble fraction; **Specific fluorescence intensity of PNS was normalized to 1 unit (mg protein)$^{-1}$; ***b.d.l.: below detection limit.

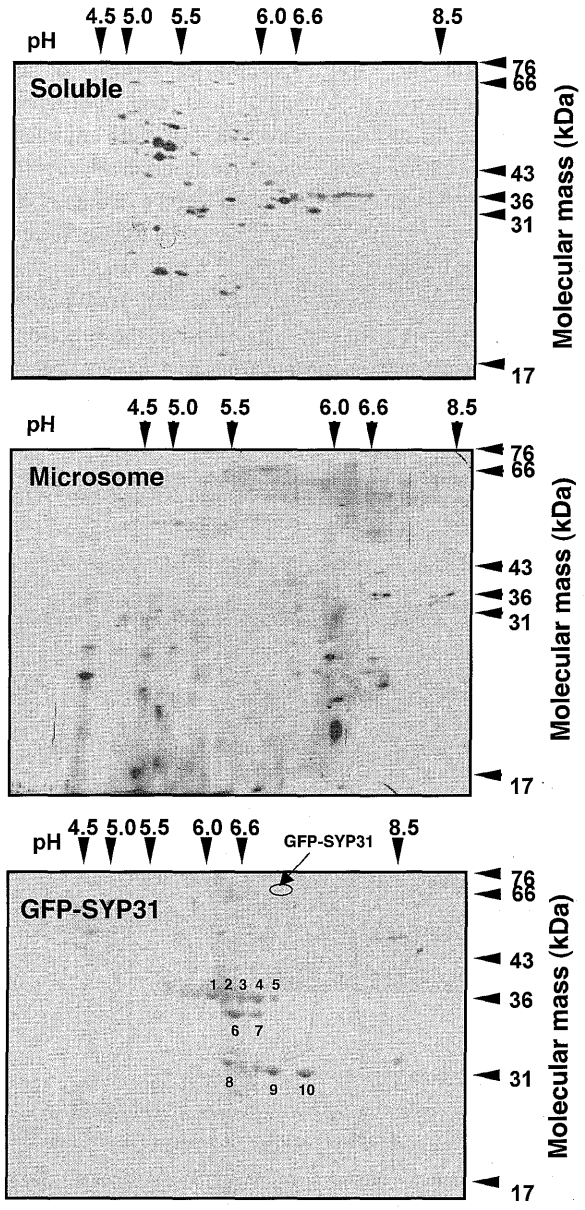

Figure 4. Separation profiles of soluble, microsomal and GFP-SYP31-labeled Golgi membrane proteins on 2D-gels. Major protein spots (no. 1–10) of the Golgi membrane fraction on the 2D-gels (GFP-SYP31) were subjected to peptide mass finger printing analysis (see Table 2). Circle represents the position of GFP-SYP31 fusion protein on the 2D-gel.

gels with CBB staining indicated that hardly if any of the Golgi membrane proteins appeared to run in the 2D-gel. The Golgi proteins might be easily aggregated during the sample preparation. Therefore, the Golgi membrane proteins were subjected to one-dimensional SDS-PAGE, followed by protein identification employing MS/MS spectrometric analyses. Edman sequencing was not performed for protein identification, since 99% of the N-terminal amino acid of Golgi proteins was blocked (Tanaka et al. 2004). We characterized 63 proteins as shown in Figure 5 and Table 3. As we expected, 70% of the identified proteins were considered to be integral membrane proteins on the basis of having predicted transmembrane domains. Trafficking-related proteins including COP complex and small GTPases were most frequently identified in the membrane fraction. Numerous GTPases (Band No. 45, 47, 48, 60) were similar to YPT1-protein, which is known to be an essential component for the ER-to-Golgi traffic (Palme et al. 1992, Fabry et al. 1993, Vernoud et al. 2003). In addition, the Golgi localized proteins, RHD3 (Zheng et al. 2004), phospholipase D (Ktistakis et al. 1995), annexin (Clark et al. 2005) etc. were identified. However, several ER localized proteins, such as HSP, protein disulfite isomerase and oligosaccharide-transferase were also identified, though ribosomal proteins were absent. There are two possibilities: (1) a part of ER and cis-Golgi have similar membrane characteristics, so that it is difficult to separate these membranes, (2) the cis-Golgi compartment actually contains sizable ER membrane proteins, since the membrane flow from ER to cis-Golgi continuously occurs. In either event, the above results clearly show that a close relationship between the ER and the cis-Golgi membranes.

Approximately 20% of the identified proteins were functionally unknown. One of these, the endomembrane protein EMP70 has also been identified in the highly purified rat Golgi membranes (Bell et al. 2001). The yeast EMP70 homolog Yer113c also has been localized to the Golgi (Huh et al. 2003). Recently, the EMP 70 family proteins were determined to be the Golgi-assigned proteins in *Arabidopsis* (Dunkley et al. 2006).

Table 2. Characterization of GFP-SYP31-labeled Golgi membrane proteins separated on 2D-gel

Spot No	Estimated Mr (kDa)	pI	Accession No	Identify	Score	Coverage (%)
1	38.4	5.9	CAD79700	Putative glyceraldehyde-3-phosphate dehydrogenase	80	25
2	38.1	6.0	CAE02009	Putative glyceraldehyde-3-phosphate dehydrogenase	185	53
3	38.1	6.6	XP_506852	Putative glyceraldehyde-3-phosphate dehydrogenase (Phosphorylating)	93	29
4	38.2	6.8	XP_506852	Putative glyceraldehyde-3-phosphate dehydrogenase (Phosphorylating)	132	42
5	38.2	7.0	XP_506852	Putative glyceraldehyde-3-phosphate dehydrogenase (Phosphorylating)	157	51
6	35.7	6.1	NP_9124221	Putative r40c1 protein-rice	138	52
7	35.8	6.8	AP005167	R40g2 protein	160	47
8	32.1	5.9	CAE76006	B1358B12.15 protein	155	55
9	31.7	6.9	CAB82853	Voltage-dependent anion channel	120	53
10	31.5	7.6	AAT39214	Voltage-dependent anion-selective channel (VDAC) protein	123	46

Tolerance: ±100 ppm

Table 3. Characterization of GFP-SYP31-labeled Golgi membrane proteins separated on SDS-gel

Band No.	Identify	Conserved domain	Calculated Mr (x 1,000)	Accession No.	Predited localization	Predicted	
						Signal	TMD
1,2,3,4	putative calcium ATPase	cation ATPase	115	AC084405	Golgi (*a), ER, PM	SP	10
5,8	similar to *Arabidopsis thaliana* putative chloroplast outer envelope 86-like protein	GTPase unknown function domain	107	XM_493929	Unknown		possible
5	putative endomembrane protein emp70 precursor	EMP70	73	AP005295	Unknown	SP	10
7	putative GPI-anchored protein		81	XM_466452	Unknown	SP	1
10	nonclathrin coat protein gamma-like protein	adaptin N , SEC21, vesicle coat complex COPI	93	AL161585	Traffic		possible
11	adaptin N terminal region, putative	adaptin N	105	AC123897	Traffic		1
10,11	putative nonclathrin coat protein gamma-like protein	adaptin N	105	AC104473	Traffic		possible
11	coatomer protein complex, beta-COP	WD40, coatmer	103	AB012247	Traffic		possible
11	unknown protein	WD40	110	AC145477	Unknown	SP	3
12,13,14	heat shock protein 90	HSP90	93	AC091774	ER	SP	possible
12	putative root hair defective 3 (RHD3)	RHD3, root hair defective 3 GTP-binding protein	90	NM_193615	Golgi (*b)		2
13	phospholipase D	protein kinase C conserved region 2, Phospholipase D. active site motifs	92	AB001920	Golgi (*c)		0
15	unknown protein	DnaJ	76	XM_472371	ER	SP	1
16	putative HSP70	HSP70	70	NM_190528	ER, Traffic (*d)		0
17	putative luminal binding protein 5 precursor	HSP70	73	XM_480535	ER, Traffic	SP	2
18	putative cell elongation protein DIMINUTO	FAD binding	64.9	AE016959	Unknown	SA	2
19	unknown protein		61	XM_479654	Unknown	SP	2
20	protein disulfide isomerase	thioredoxin	56.9	AY987391	ER	SP	possible
21	cinnamate-4-hydroxylase		56.9	AC136224	Golgi (*e)	SP	2
22	putative beta -1,3-glucanase	glycosyl hydrolases family 17	60.1	XM_478552	Golgi (*f), PM	SP	possible
23	putative dolichyl-di-phosphooligosaccharide-protein glycosyltransferase	dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48kD subunit	48	XM_478552	ER	SP	2
24	EF-1 alpha	elongation factor Tu GTP binding domain	49	D63581	Cyt		0
25	phosphate/phosphoenolpyruvate translocator	transporter superfamily	45.1	AAB40648	Mit		6
26	OSJNBa0006M15.17	ER to Golgi transport-related protein, ERGIC3-like	43.5	XM_472721	Golgi,Traffic	SA	2
27	unknown protein	ER to Golgi transport-related protein, ERGIC3-like	43.1	XM_475457	Golgi,Traffic	SA	2
28	P0425G02.25	protein kinase C interacting protein	40.6	NM_191060	Traffic (*g),Cyt		2
29	putative glyceraldehyde-3-phosphate dehydrogenase	GAPDH	38.4	CAD79700	Traffic (*h), Cyt		0
30	putative glyceraldehyde-3-phosphate dehydrogenase	GAPDH	38.1	AL606998	Traffic, Cyt		0
31	putative sec61	secY, preprotein translocase subunit SecY	37.4	AP005313	ER	SA	5
32	PREDICTED OJ1791_B03.34 gene product	GAPDH	36.7	XM_506852	Traffic, Cyt		0
33	osr40c1		39.3	X95402	Unknown		0
34	osr40g2		39	XM_479571	Unknown		0
35	Toc34-1 protein	TOC	36.6	AJ245968	Chl		1
36	putative membrane protein	hydrolase	36.2	XM_479104	Unknown	SP	1
37	putative annexin		35.9	AP004727	Golgi (*i), PM		0
38	putative prohibitin		31.5	XM_477318	Mit	SP	possible

Table 3. Continued

Band No.	Identify	Conserved domain	Calculated Mr (x 1,000)	Accession No.	Predited localization	Predicted	
						Signal	TMD
39	33-kDa secretory protein		30.4	XM_479978	Cargo	SP	possible
40	voltage dependent anion channel		29.6	XM_475771	Golgi (*j), Mit		1
41	putative vesicle-associated membrane protein 725	Synaptobrevin/VAMP	27.6	XM_483759	Traffic		1
42	putative GTP-binding protein	RAB11	24.3	XM_475070	Traffic		0
43	putative cyclophilin		24	AP003621	Cargo	1	1
43	putative GTP-binding protein	RAB11	24.2	XM_475070	Traffic		0
43	PREDICTEDP0471A11.32	crystallin	24	XM_506984	Golgi (*k)		0
44	expressed protein		24.2	XM_470465	Unknown		0
45	GTP-binding protein	YPT1	24	AAB28535	Traffic		0
46	putative COP coated vesicle membrane protein	EMP24	23.6	XM_469701	Traffic	SP	1
47	ras-related GTP binding protein possessing GTPase activity	YPT1	23	AAB28535	Traffic		0
48	putative GTP-binding protein YPTM2	YPT1	22.6	XM_467097	Traffic		0
49	putative GTP-binding protein RAB	RAB7	23	XM_475712	Traffic		0
50,51	putative TOM20	protein translocase	22.6	AP006843	Mit		1
52	unknown protein		21.1	XM_467487	Unknown		2
53,54	OSJNBa0027p08.20	microsomal signal peptidase	21.1	XM_472655	Unknown		2
55	unknown protein		21.9	XM_474289	Unknown	SA	4
56,57,58	translocon-associated protein beta	TRAP-beta	20.2	NM_183453	Traffic	SP	1
56,61,62	putative class 1 low molecular weight heat shock protein	HSP20 small heat shock protein	17.9	NM_187465	ER		possible
57,58	translocon-associated protein beta	TRAP-beta	20.8	XM_493875	Traffic	SP	2
59	calcium binding protein	EF-hand	19.3	XM_473766	Unknown		2
60	small GTP binding protein	YPT1	21.6	D01027	Traffic		0
60	YPTV3	YPT1	22.3	P36862	Traffic		0
63	unknown protein	Tim17	15.7	XM_467481	Unknown		2
64,65	amino acid selective channel	Tim17	15.6	AC079356	Mit		2
66,67	unknown protein	integral membrane protein	19.1	XM_476905	Unknown	SA	1
68	unnamed protein product	ATP synthase gamma	14	XM_463395	Mit		1

The masscot search was carried out with ±0.5 Da peptide MS tolerance and ±0.3 Da fragment MS tolerance. The proteins hitted by masscot search were further judged with reference to molecular mass estimated by SDS-PAGE. Unknown protein without any characteristic domain was not listed. Chl, chloroplast; Cyt, cytosol; ER, endoplasmic reticulum; Mit, mitochondria; PM, plasma membrane. (*a) Ordenes et al. (2002); (*b) Zheng et al. (2004); (*c) Ktistakis et al. (1995); (*d) Joglekar and Hay (2005); (*e) Sato et al. (2004); (*f) Mauch and Staehelin (1989); (*g) Tisdale (2003); (*h) Tisdale (2001); (*i) Clark et al. (2005); (*j) Buettner et al. (2000); (*k) Gangalum et al. (2004). Signal peptides and the number of transmembrane domains were predicted according to algorithms, SignalP, PSORT, HMMTOP, SOSUI, TMHMM, and Tmpred. SP, signal peptide; SA, signal anchor; TMD, transmembrane domains.

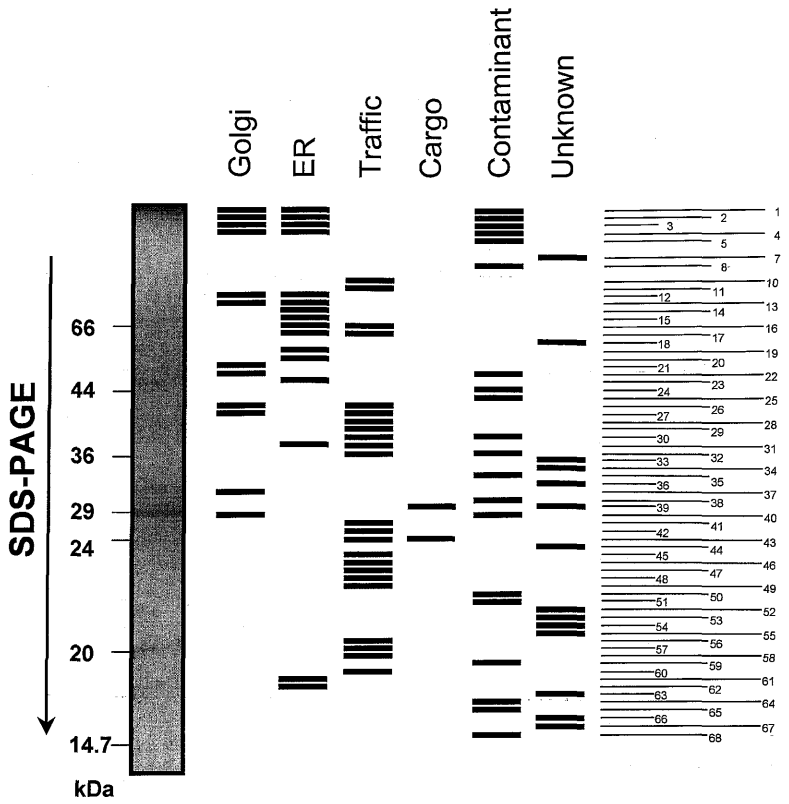

Figure 5. Separation profile and identification of GFP-SYP31-labeled Golgi membrane proteins after SDS-PAGE. SDS-gel was sliced into pieces 1 mm in width, and each gel slice (1 to 68) was subjected to MS/MS analysis (see Table 3). Identified proteins were categorized to Golgi, ER, Traffic, Cargo, Contaminant and Unknown.

The EMP70 is a multiple transmembrane-containing protein similar to Ca^{2+}-ATPase. In addition to the function of EMP in the plant Golgi, its Golgi retention mechanism is also totally unknown.

The Golgi complex is made up of approximately 1,000 proteins. About 200 Golgi proteins have been identified from a variety of tissues from several animal species (Taylor et al. 2000). A proteomic analysis of proteins from rat hepatic Golgi fraction has been reported (Bell et al. 2001). A total of 81 proteins have been identified. They include Golgi-resident enzymes, Golgi lectin, anterograde cargo, KDEL receptors, p24 family members, SNAREs, RABs, and ARF-GEF. Proteomic analysis of *Arabidopsis* Golgi has been performed by using the localization of organelle proteins by isotope tagging (LOPIT) technique (Dunkley et al. 2006). The 89 proteins assigned by LOPIT to the Golgi apparatus largely belong to three main classes: predicted glycosyltransferases, EMP70 proteins, and putative methyltransferases. In this study, the putative galactosyltransferase gtl6 was used as the Golgi apparatus marker, so that the Golgi proteins assigned by LOPIT were assumed to be the whole Golgi or the medial/trans-Golgi residents. The present study revealed the presence of a set of RAB (YPT1) family in rice cis-Golgi. However, SNAREs and the oligosaccharide-trimming enzymes were hardly detectable in our experimental systems. These proteins may be minor components in the rice Golgi system. Further exhaustive analysis of Golgi membrane proteins is required for clarifying the plant Golgi proteome in detail.

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